ISOLATION, MAINTENANCE, AND PURE CULTURE MANIPULATION OF ECTOMYCORRHIZAL FUNGI

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INTRODUCTION

Isolation and experimental manipulation of ectomycorrhizal fungus cultures have been critical in developing all phases of ectomycorrhizal research. In vitro culture studies provide insight into the basic biology and processes of the fungal symbionts, e.g. growth responses to varying pH, temperature, and moisture regimes, mineral and carbohydrate physiology, and production of enzymes and hormones. Cellular interactions between fungus and host as well as with other microorganisms, including pathogens, can be critically examined in vitro. Isolation and comparative study of diverse fungal species and isolates within species provide a basis for selecting specific isolates for artificial inoculation of nursery stock (28, 71).

A tremendous body of literature exists on pure culture studies of ectomycorrhizal fungi; we will not attempt to review all aspects. Our purpose is to describe several techniques common in study of ectomycorrhizal fungus cultures. Isolation, maintenance, nutritional requirements, ectomycorrhiza synthesis, and basic precepts will be stressed. Variations of the methods described may work equally well to meet unique or exacting circumstances of individual researchers. Alternative techniques are also described in many of the references cited and in recent mycology texts (26, 72).

ISOLATION OF ECTOMYCORRHIZAL FUNGI

Ectomycorrhizal fungi are most commonly isolated from sporocarp tissue but can also be isolated from surface sterilized ectomycorrhizae, sclerotia, rhizomorphs, and, in some cases, from sexual spores. Sporocarp isolation is generally preferred because species can be identified and little pretreatment of fungal material is required. Ectomycorrhizal fungi can differ strongly in ease of isolation from sporocarps and growth in culture. However, consistent trends in isolatability are evident within certain taxonomic groupings. For example, most species of Suillus and Rhizopogon are easily isolated and grow well in culture. In the genus Amanita, species in subgenus Amanita are more easy to isolate and grow in culture than species of subgenus Amanitopsis. Only a few species have been successfully isolated in some genera, e.g. Russula, and in still others no successful isolations have been made, e.g. Gomphidius. Many species within the following genera can be routinely isolated from sporocarps: Alpova, Amanita, Astraeus, Boletus, Cortinarius, Fuscoboletinus, Hebeloma, Hymenogaster, Hysterangium, Laccaria, Lactarius, Leccinum, Melanogaster, Paxillus, Pisolithus, Rhizopogon, Scleroderma, Suillus, and Tricholoma. The inability to isolate several species stems from ignorance on the exacting nutrient requirements which they likely derive from hosts. Further nutritional studies are needed to bring these recalcitrant species into culture.

Collection of sporocarps.--Collectors should familiarize themselves with the seasonal fruiting of fungi so that sporocarps in good condition and in various developmental stages can be collected. Young sporocarps are preferred for direct isolations. Fully matured specimens should also be collected, however, so that species can be reliably determined. Place sporocarps into waxed paper bags or wrap in waxed paper to retard drying (avoid plastic bags because sporocarps deteriorate rapidly in a "non-breathing" container). In the case of mushrooms, cut the stem from a mature specimen in good condition, place the cap over a white card, cover with waxed paper, and lay cap over card flat in the collection container to obtain a spore print. Record field notes on macroscopic sporocarp characters (pay attention to color changes when specimens are bruised) and all

potential hosts in the vicinity immediately upon collection. It's best to familiarize oneself ahead of time with critical field characters needed to identify anticipated fungus collections as well as the ectomycorrhizal hosts in the area. Seek prior advice or, even better, participation of a taxonomic mycologist.

Isolations should be done as soon as possible after collection; best results are often obtained when done immediately in the field (53, 71). Properly stored sporocarps of many species, however, can yield successful isolations after several days. While in the field, avoid freezing, heating (direct sunlight) or drying of sporocarps. If isolations are to be attempted one or more days after collection, the specimens should be refrigerated (3-5°C).

Accurate identification of fungi is critical to interpreting research results. If identifications are uncertain, request confirmation by a mycologist familiar with the group in question. We stress that properly dried voucher specimens must be prepared for all isolates and accessioned into an active mycological herbarium for future reference (1); include the specimens from which the isolates were actually obtained.

Collections of ectomycorrhizae, rhizomorphs and Cenococcum sclerotia.-- Purpose and circumstance may dictate the selection and collection of ectomycorrrhizae for direct isolation. Select roots with as little adhering debris as possible. Ectomycorrhizae in some substrates, e.g. rotten wood, are cleaner than in others, e.g. mineral soil. Collect enough material for 100-200 isolation attempts for each desired ectomycorrhiza type. Isolate as soon as possible after collection. Fine roots can dehydrate rapidly but preserve well if placed together with a little moist soil, humus, or moss in a "non-breathing" container (tightly closed can, jar, or plastic bag). If necessary, refrigerate root samples (3-5°C) until used. Rhizomorphs should be collected and stored similarly.

Cenococcum geophilum Fr. is most easily isolated from its hard black sclerotia (69). The long-lived sclerotia abound in most stands of ectomycorrhizal hosts and can be extracted from soil samples any time of year. To collect sclerotia, rake away the upper humus and sample the organic-mineral soil interface to a depth of 10-15 cm; at least five subsamples (trowel fulls) should be collected around the immediate vicinity of the host plant to yield 1 to 2 liters of soil. Take the samples back to the laboratory and, if neccessary, store them in tightly closed, "non-breathing" containers under refrigeration until sclerotia are extracted. Isolation should be attempted as soon as possible, but samples can be stored for many weeks without measureable loss of sclerotium viability.

Preparation for isolation.--A clean work area with still air is important to minimize contamination and generally is as good as specially designed isolation hoods. In the field, we have had good success using a small portable isolation chamber constructed of white-painted plywood with a slanted, transparent, plexiglass shield on top (Fig. 1); a rectanglular 15 X 40 cm opening in front allows easy hand entry and maneuverability within the box for flaming metal tools, tissue extractions and transfers onto nutrient agar. This portable chamber is easily set up on picnic tables in campgrounds, on stumps or logs, or on tables in travel accomodations. Any bench space in a clean laboratory is ideal for isolating. However, field material often harbors pests such as mites which can enter and consequently contaminate culture vessels. Thus, field collections should not be brought into areas where stock and experimental cultures are kept.

Necessary tools and materials include fine tipped, heat-sterilizable scalples, transfer needles, forceps, alcohol or gas lamps, and a general disinfectant such as 5% sodium hypochlorite or 95% ethanol. Abundant test tubes or petri plates with nutrient agar should be available; tubes are less subject to contamination than plates. At the Corvallis Forestry Sciences Laboratory, we routinely use modified Melin-Norkrans nutrient agar (MMN) (Table 1) with dextrose as the carbon source and potato dextrose agar. Addition of antibiotics can prove useful for reducing contamination, especially for isolations from roots.

Isolation from sporocarp tissue.—If available, select young sporocarps free of rot and insect (larvae) damage. Brush off adhering debris, especially from the stipe base; if the base is difficult to clean, cut it off. For mushrooms, cut a shallow (1-2 mm) slit across the middle of the cap surface and along the length of one side of the stipe. On tuber-like hypogeous sporocarps, the initial shallow cut should circumscribe roughly one-half of the fruit body. Do not cut through the entire specimen to expose interior tissue because this will drag surface contaminants into the cuts. To expose interior tissue, gently pull apart the sporocarp along the initial shallow cuts, using fingertip pressure; placing a fingernail into the initial cut will lend more leverage for breaking sporocarps composed of tough tissue. Quickly scan the exposed interior surface for areas of sound tissue free from contact with obvious contaminating sources. With a fine tipped scalpel (flame sterilized and cooled), cut and loosen small pieces (2-5 mm cubes) of sound tissue. Transfer the tissue explants with the scalpel or a sterile transfer needle directly onto nutrient agar in tubes or plates.
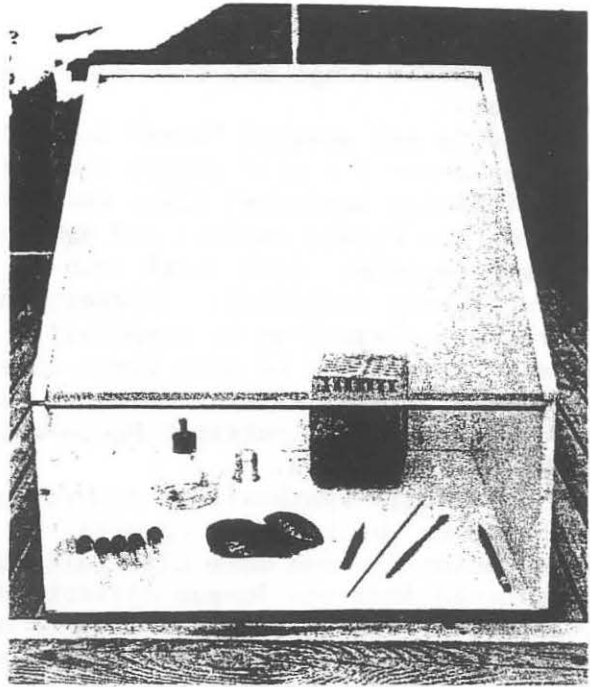

Fig. 1. Portable culture chamber for field isolations. Plywood frame and clear, plexiglass top reduce air movement; open front provides easy hand access.

Because sporocarp tissues are often strongly differentiated, attempt isolations from two to three locations on the sporocarps, e.g. cap and stipe (tissue from over the gills often seems to grow best). For most hypogeous fungi, such as Rhizopogon spp., the center of the gleba is best for isolation. With Scleroderma spp., the interior of the leathery peridial tissue is good. Isolations should be attempted from more than one specimen for greater assurance of success. The number of individual tissue transfers depends on the importance of obtaining an isolate and one's experience in isolating that particular species. For example, with fresh, larva-free sporocarps of Rhizopogon or Suillus spp., 6 to 10 tissue transfers are generally enough. If you are not familiar with the fungus species and an isolate is critically needed, 10 to 20 or more attempts should be made. Tissue transfers are routinely incubated at 20°C, but room temperature is often adequate.

After 3 to 4 days, begin observations under a stereomicroscope for initial fungus growth and contamination. Fungi which are easily isolated and grow well in culture produce visible mycelial growth 4 to 7 days after isolation; other fungi may take 2 to 6 weeks to show any sign of growth from the tissue. Most species "bush-out" in all directions from the tissue explant. Others, like Laccaria spp., grow only submerged in the agar or prostrate on the agar surface. Cultures should be frequently observed over the first few weeks and characteristics of emerging colonies and presence of contaminating microbes recorded. Check particularly for uniformity in culture characteristics of the mycelia emerging from the various tissue explants. Several uniform cultures emerging from different explants provide initial evidence of sucessful isolation. After the suspected ectomycorrhizal fungus is established on the agar substrate and no contaminants are visible, mycelium from the new colony edge should be aseptically transferred with a transfer needle onto fresh nutrient agar to set-up a stock culture. Ectomycorrhizal fungi that grow well in culture will be ready for initial transfer after 3 to 4 weeks. Slow growing fungi may take 2 to 4 months before they are ready for transfer and are easily lost in subsequent transfers. Tissue explants

of recalcitrant fungi may show no contamination, but new hyphae never emerge from the tissue.

Bacteria and several "weed" fungi (Trichoderma, Aspergillus, Penicillium, and yeast spp.) are the most common contaminants and are easily detected by presence of a glistening bacterial slime or characteristic fungus conidia. Contaminants can swamp the tissue explant and agar substrate before the ectomycorrhizal fungus can begin to grow. Some fungi such as Laccaria spp. routinely yield bacterial associates upon isolation. However, the fungi often out-grow the bacteria and cultures are cleaned-up by aseptically removing a bit of mycelium from the colony edge and transfering it onto fresh substrate. This may have to be done repeatedly until bacteria are absent. Cultures with questionable bacterial contamination should be grown in a nutrient broth wherein proliferation of any bacteria present will cloud the liquid.

A mycorrhiza synthesis is needed to confirm that a species isolated for the first time is the intended species and if it forms ectomycorrhizae. This determination is even more difficult when two or more different-appearing fungi are isolated from one fungus collection. Familiarity with the cultural characteristics of the fungus species or comparison to known cultures of that fungus greatly aid culture confirmation. Clamp connections indicate that the fungus is a basidiomycete, but not all basidiomycetes form clamps. Culture descriptions of many ectomycorrhizal fungi are available in the literature, as are suggested ways to identify ectomycorrhizal fungi from cultural characteristics (59, 65, 72). As emphasized later, a pure culture mycorrhiza synthesis provides the strongest evidence of isolation of an ectomycorrhizal fungus.

Isolation from ectomycorrhizae.--Many ectomycorrhizal fungi can be isolated from their ectomycorrhizae and occasionally from large rhizomorphs, provided they can grow on agar media. Best results are obtained when fresh ectomycorrhizae, initially free of adhering debris, are treated immediately upon collection (3, 73, 76, 79, 80). Isolation success from individually treated ectomycorrhizae varies considerable between different fungi but is usually less than 20% and frequently less than 5%. Thus, isolation should be attempted from a large number of mycorrhizae.

After collection, ectomycorrhizae are sorted and the cleanest ones selected. The following basic treatments are then recommended by Zak (73, 76, 79, 80): 1) rinse the roots under tap water to remove loosely adhering debris; 2) place rootlets into a perforated plastic vial and shake vigorously for 3 min in a mild detergent solution (sonication in a water bath during or prior to step 2 further aids in loosening surface debris); 3) rinse off detergent with tap water; 4) soak for 4 min in 100 ppm mercuric chloride or 5-20 sec in 30% hydrogen peroxide; 5) rinse immediately with 2 liters of sterile distilled water and 6) aseptically transfer individual ectomycorrhizae (or rhizomorph pieces) onto nutrient agar in test tubes or plates. If results are poor, try varying the length of soaking in the surface sterilant. A variation on the soaking time is to put large numbers of root tips into the surface sterilant in a sterilized Petri plate and transfer some to sterile distilled water in another plate at intervals, starting at 2 and ending at 6 min, with the expectation that some point in that time span will prove to be just right. See Zak (73, 76, 79, 80) for greater detail on experimental procedures for this subject. Other methods of surface sterilization are described by Chu-Chou (3) and Slankis (63).

Surface sterilized ectomycorrhizae are incubated and growth is checked as described for isolation from sporocarps. Emergent growth of the mycorrhizal fungus usually occurs 2 to 4 weeks after treatment. If the identity of the fungus symbiont is known, e.g., reisolation of a fungus used in experimental inoculations, then it is relatively easy to compare culture characteristics to stock cultures and confirm reisolation. If the fungus symbiont is not known, comparisons with cultures taken from sporocarps found in the same vicinity, examination for clamps connections, and mycorrhizal syntheses must be done to confirm its ectomycorrhiza forming ability and indicate possible identity.

Isolation from Cenococcum sclerotia.--C. geophilum sclerotia are easily extracted from soil samples as described by Trappe (69). Small soil samples

(25-50 ml) are placed in an evaporating dish and wetted to make a slurry with free water on its surface. The dish is gently hand swirled so the low-density soil fraction containing sclerotia floats to the surface and can be poured off into another dish. Large volumes of soil can also be wet sieved through a 0.344 mm screen. Final fractions are placed in water under a stereomicroscope and the black sclerotia removed with forceps.

Sclerotia are treated similarly to ectomycorrhizae for isolation. Select large, clean sclerotia, free of obvious cracks or breakage. Remove small adhering debris by sonication in a water bath for 10 min. To surface sterilize, Trappe (69) recommends soaking for 10 to 20 min in 30% hydrogen peroxide. However, other surface sterilants work well and one may want to vary soaking time and sterilants to achieve maximum success. Rinse sclerotia in sterile water and aseptically transfer onto nutrient agar (MMN works well). As with ectomycorrhizae, attempt isolation from 30 or more sclerotia per soil sample. Incubate as previously described. Near-white to gray hyphal tips usually appear after 2 weeks. Established colonies are easily recognized as coarse hyphae which become jet black behind the tips (16, 69, 70).

Isolation from sexual spores.--Regardless of technique, ectomycorrhizal fungi are rarely isolated from basidio- or ascospores and so will not be covered in detail. Fries (8) has recently developed a promising technique involving germination of spores in the presence of activated charcoal to remove inhibitors plus the yeast Rodotorula glutinus (Fries) Harrison, which acts as a stimulant. Other Fries studies (9, 10) have also shown that mycelia of the same fungus and living roots of axenic seedlings can further stimulate spore germination. Readers are referred to Fries (8, 9, 10) for more details on experimental procedures and to Palmer (57) for information on spore drop techniques.

MAINTENANCE IN CULTURE

When ectomycorrhizal fungus cultures are needed over several years, stock cultures should be maintained separately from working cultures. Stock cultures are most commonly stored on nutrient agar slants in test tubes under refrigeration (3-5°C). At the Corvallis Forestry Sciences Laboratory we store four slants (13 X 100 mm screw-capped glass tubes with 3 ml agar) for each fungus isolate in a refrigerator at 3°C, most commonly on MMN nutrient agar but sometimes also on potato dextrose agar or both. Each isolate is transferred every 3 to 4 months. Transferred cultures are incubated at 20°C (usually for about 1 to 2 weeks or until the mycelium is firmly established on the agar) and then immediately placed in cold storage until the next transfer period. With this regime, the cultures grow slowly during most of their storage life and thus have less chance to modify their growth physiology as they sometimes do when cultures are kept in a constantly active growth state. Not all ectomycorrhizal fungi store well under such conditions; some prefer warmer incubation temperatures or more frequent transfers. Experiment with variations of the procedure to find the best storage conditions for troublesome species.

Marx and Daniel (31) emphasize that many ectomycorrhizal fungi change growth habits and may lose their ability to form ectomycorrhizae after long storage periods in a growing state. They found, however, that several ectomycorrhizal fungi can be stored for up to 3 years and probably longer in sterile cold water. Fungi are first grown on nutrient agar in plates from which 8 mm diam agar plugs are cut from the colony edge; 10 to 15 plugs are then aseptically transferred into 2.5 X 15 cm glass screw-capped test tubes containing 25 ml sterile distilled water and stored at 5°C in darkness. When needed, the fungus is retrieved by aseptically removing and transferring a mycelial disk onto fresh nutrient agar. This technique will not work with all fungi, so one must test each isolate for retrievability from this system before obligating stock cultures to this storage method.

Thorough records must be kept for each isolate maintained in stock cultures. Important data include identification number, isolation source (sporocarp, ectomycorrhiza, rhizomorph, etc.), date of isolation, species identifier and

voucher (herbarium) number, associated hosts in the immediate vicinity of collection, location of collection and habitat type, experimentally determined hosts, and best storage medium and conditions. If isolates are used in experimental inoculations and then reisolated, the new isolate should be given a different identification number and the experimental conditions noted.

Mycorrhiza researchers regularly share fungus cultures for various research purposes. Firmly sealed and carefully packaged cultures can easily be sent long distances by air mail. Be sure to include complete background data for each isolate so that the receiver can cite this information in subsequent publications. Check for possible quarantine restrictions before mailing cultures abroad.

GROWTH IN PURE CULTURE

Nutrients.--The hexose, d-glucose, is used by almost all ectomycorrhizal fungi. Therfore this sugar is included as the sole or principal carbon source in synthetic media and as the principal or supplementary source in semisynthetic substrates. The two most commonly used concentrations are 5 g/l and 20 g/l. Other 6-carbon compounds are not utilized by as many fungi. Sorbose is mostly nonutilizable. Sugars with 3-, 4-, and 5-carbons are not used, or growth is poor. Among disaccharides, cellobiose, maltose, trehalose, and sucrose are satisfactory for many isolates. However, sucrose may dissociate to glucose and fructose at high temperatures, low acidity, or both. A few isolates grow well on trisaccharides or 3- to 6-carbon alcohols. However, phosphate ions at concentrations above those commonly used may reduce growth on some groups of carbohydrates. In general, soluble polysaccharides are more utilizable than the insolubles and polysaccharide derivatives. A few organic acids or their salts, e.g. citric, fumaric, malic, propionic, and succinic, support growth for a few isolates. Acetates are usually inhibitory. (See references 6, 11, 20, 21, 55, and 58).

Ammonium chloride (0.5 g/l) and ammonium nitrate (1.0 g/l) are most commonly used as supplemental nitrogen sources in semisynthetic nutrient solutions. In synthetic formulae, ammonium tartrate (0.5, 1, and 5 g/l) is most used. Other ammonium salts from 0.25 to 1.0 g/l and asparagine at 1.0 g/l are satisfactory. Nitrates are mostly nonutilizable. Consequently, ammonium nitrate is probably a good choice for initial attempts to isolate or culture a new species. In any case, a rapid rise or fall in pH can be expected with use of inorganics if the solution is unbuffered. That ammonium tartrate is a buffer, as is potassium monobasic or dibasic phosphate, probably explains its preferential use as the nitrogen source. However, experimental procedures that necessitate exclusion of any additional and possibly utilizable carbon usually precludes use of ammonium tartrate, ammonium salts or other organic acids, asparagine and other proteinaceous compounds. In addition, many of these substances are growth regulators, which make them undesirable for use in growth regulatory studies. (See references 20, 24, 37, 39, 40, 48, and 55).

Macronutrients other than nitrogen and carbon have not been investigated with specific intent to determine exact quantities or essentialities. The same applies to micronutrients. No doubt this reflects the complex and time-consuming procedures required. Experience indicates that phosphorus, potassium, sulfur, and magnesium must be present in relatively large amounts and copper, iron, manganese, molybdenum, and zinc in small amounts for growth of nearly all fungi. Each of the macronutrients is commonly added to both semisynthetic and synthetic formulae. Potassium and phosphorus are added as potassium di-hydrogen phosphate in either the 0.5 g/l or 1.0 g/l quantity. Magnesium and sulfur are added as magnesium sulfate ($MgSO_4 \cdot 7H_2O$) at 0.5 g/l in nearly every case.

At least one micronutrient is added to most semisynthetic solutions and several to most synthetic ones, more commonly as single chemicals rather than composite solutions. Iron in the form of ferric chloride near the level of 0.005 g/l is common although 1.0 - 0.3 ppm of iron is considered to meet the essential amount. Unfortunately, ferric chloride will diffuse into most types of glass, which makes storage in stock solution relatively short-term. Consequently ferric

nitrate, ferric sulfate, or ferric citrate is often substituted, but each adds a small quantity of a macronutrient. Inorganic iron salts precipitate from solutions in increasing amounts as the pH rises above 4.0. Organic ferric salts or inorganics accompanied by an organic acid will stay in solution. At the Corvallis Forestry Sciences Laboratory we routinely incorporate the chelated iron product Geigy's Sequestrene (sodium ferric diethylenetriamine pentaacetate) into synthetic media at 0.02 g/l with good results. Zinc is added to nearly all semisynthetic and synthetic formulations as zinc sulfate commonly at levels between 1 and 5 mg/l. Conversely, inclusions of copper and manganese, which are probably essential for at least some isolates, are infrequent. Calcium as calcium chloride is more often added than is manganese. Quantities vary between 50 and 200 mg/l with 55.5 mg/l (5 ml of a 0.1 molar solution) most frequently used. Such anions as calcium, iron, and zinc become unavailable at a pH near 7.4, but an elevated pH is infrequent with these acidophiles. (See references 21, 27, 55, and 57).

In regard to vitamins and growth regulators, many ectomycorrhizal fungi are heterotrophic for thiamine; a few are heterotrophic for biotin. Neither vitamin is regularly added to semisynthetic media. Thiamine is added to most synthetic solutions usually at 50 µg/l but varies between 1 and 100 µg/l. Biotin is also added to some solutions before sterilization. The usual rate is 5 µg/l or 10 µg/l. Both should be added if the fungus has not previously been cultured or isolated. Stimulation of growth by other growth regulators is frequent; growth suppression is unlikely at the concentrations used, i.e. less than 1.0 g/l. Inoculation of single compounds and natural mixtures, e.g. casein hydrolysate, frequently increase growth but rarely to a significant level. Duplicable formulae of amino acids reported to stimulate casein hydrolysate (46) and vitamin complexes (18, 46) or both at 1.0 mg/l may initiate culture or increase growth. (See references 20, 39, 41, 45, 55, and 62).

Environmental Conditions.--In axenic culture, responses are isolate specific, but most fungi are acidophilic (pH optima 4.5–5.0, ranges 3.5–6.0), aerobic (require gaseous oxygen), mesothermal (optima between 18°C and 25°C), and photoinactive (no induction of zonation or pigmentation). In the usual closed-container culture air movement and relative humidity are uncontrollable. Osmotic pressure is initally a function of ionized and soluble compounds per unit water but subsequently varies with absorption, secretion, and lysis of mycelium. Outside of pH, our standard conditions (temperature 22°C, relative humidity 50%, constant light provided by equal mixtures of cool white and daylight fluorescent bulbs) indirectly affect culture chamber conditions. Stabilization of pH with buffers is possible but will complex some experimental procedures, especially if phosphates or organic acids or salts are involved. (See references 11, 14, 22, 27, 33, 37, 47, 49, and 55).

Media.--Semisynthetic formulations are most commonly used for propagation, inoculum preparation, storage on agar, fruiting, and other experimental procedures. The most common has been the Modess (49) modification of the original Hagem (15) or a variant of this formula (see Table 1). The MMN solution (Table 1) represents a Marx (27) enrichment of a Melin-Norkrans formula. The original solution (54) was a synthetic solution (no malt extract) with only 25 µg of thiamine and 2.5 g of glucose. Formulae used in eastern Europe often replace or augment malt extract with casein or yeast hydrolysate or both. (See references 4, 18, 23, and 47).

Synthetic nutrient media are used primarily to examine carbon and nitrogen nutrition and mycelial growth regulation by various compounds. Formulations are numerous; two are tabularized in Table 1. The Melin and Rama Das (46) formula included neither the synthetic casein hydrolysate nor the vitamin mixtures, and most users do not indicate inclusion or exclusion in experiments. With reference to "see Text" listed in Table 1, a micronutrient solution at 2 ml/l is always included in our solution: dissolve $Fe(NO_3)_3 \cdot 9H_2O$ at 1.45 mg, $ZnSO_4 \cdot 7H_2O$ at 0.88 mg, and $MnSO_4 \cdot H_2O$ at 0.41 mg in 700 ml of distilled water, acidify with H_2SO_4 to give a clear solution, and dilute to 1,000 ml with distilled water (57). (See references 21, 24, and 33).

TABLE 1. Semisynthetic and synthetic nutrient formulae commonly used for propagation, inoculum preparation, experimentation, and storage of ectomycorrhizal fungi in axenic culture

NUTRIENTS	SEMISYNTHETIC		SYNTHETIC	
	"Hagem" (Modess 1941)	MMN (Marx 1969)	Melin & Rama Das (1954)	Palmer & Hacskaylo (1970)
Malt Extract	5.0 g	3.0 g		
d-Glucose	5.0 g	10.0 g	20.0 g	5.0 g
NH_4Cl	0.5 g			0.5 g
$(NH_4)_2HPO_4$		0.25 g		
$(NH_4)_2C_4H_4O_6$			0.5 g	
KH_2PO_4	0.5 g	0.5 g	1.0 g	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g	0.15 g	0.5 g	0.5 g
$CaCl_2$		0.05 g		
$FeC_6H_5O_6 \cdot 3H_2O$			0.5 ml (1% solution)	
$FeCl_3$	10 drops (1% solution)	1.2 ml[b] (1% solution)		
NaCl		0.025 g		
$ZnSO_4$			0.5 ml[a]	
Biotin				5.0 µg
Thiamine			1.0 µg	1.0 mg
Thiamine HCl		100 µg		
Micronutrients				(see Text)
Distilled H_2O	1,000 ml	to 1,000 ml	to 1,000 ml	to 1,000 ml
pH after autoclaving	4.6 - 4.8	5.5 - 5.7	Unlisted	4.5 - 4.7

[a] 1:500 Zinc in aqueous solution.
[b] Zak (unpublished) substitutes 0.02 g Sequestrene (Geigy).

Use of natural formulations is infrequent. However, Zak (74) regularly used the Lacy and Bridgmon (19) potato dextrose (PD) and Russian workers, the Voznyakovskaya and Ryzhkova cabbage dextrose (CD) as reported by Lobanow (23). The formula for the PD is 1) rehydrate 22 g of dehydrated potatoes in 178 g distilled water, 2) add 20 g d-glucose followed by agar if desired, and 3) stir well, 4) utilize all but potato sediment, and 5) autoclave for 20 min. Formula for the CD is: 1) weigh 100 g cabbage 2) cook for 10 min in 1 liter of tap water (we use distilled or deionized water), 3) filter, 4) adjust pH to 5.0-5.6, 5) add 3% glucose, 6) add a 0.5% solution of thiamine at the rate of 1.0 ml/10 liters of nutrient solution, 7) add agar if desired, and 8) autoclave the solution.

Techniques.--The common substrate is water that has been singly or doubly distilled or deionized or purified by some combination. Gelling with 2 + 0.5% agar of various purities has advantages for routine propagation. Growth of some fungi can be initiated or accelerated in aerated particulate substrates such as mixtures of vermiculite + peat. The pH of vermiculite tends to rise with time but can be stabilized at least in uninoculated mixtures (32, 61).

Containers made of the same material, even from the same lot of plastic, provide relatively homogenious conditions during prolonged experiments. At the Madison Forest Products Laboratory we prefer borosilicate glass test tubes (16 x 150 mm), Petri dishes (100 x 20 mm), and narrow mouth Erlenmeyer flasks (300 ml). Large Petri dishes enable inclusion of additional agar and delay drying. The flasks capped with 50 ml Griffin low-form beakers stablilize gas exchanges, do not rattle much on shakers, and provide good surface aeration. Unscratched

borosilicate glass also provides uniform tranmission of quantity as well as wave-lengths of light irradiated from a constant source.

Inoculation of agar plugs or chunks to fresh nutrient agar usually gives commensurate growth if there is no film of liquid water at the implantation point. Any method used to inoculate a liquid substrate has limitations. Specific techniques often work well only with certain isolates. In nearly every case, the initial growth lag phase is shorter for floating than for submerged inoculum, even in those fungi that produce asexual reproduction structures such as conidia or oidia. We prefer the "fuzzing" technique if mycelial dry weight is to be measured: 1) cut plugs from agar at the edge of a colony (a 5 mm diameter cork borer is excellent), 2) transfer to fresh agar with the mycelium on the upper side of the plug, 3) keep plates at 21 $\pm$ 2°C, 4) observe at least once each day for horizontal extension of hyphae all around the elevated periphery, and 5) remove and float plug on the liquid substrate. Readers should be aware that at some time after "fuzzing," the hyphae will reflex. Thus, for isolates that project hyphae horizontally, a number of plugs much in excess of the number needed should be prepared. For nutrient studies in which the absence of key nutrients is essential, cover slips covered with a complete nutrient agar can be placed upon agar lacking the experimental nutrient, and the fungus is inoculated onto the cover slip's agar surface. Some hyphae will then grow out over the deficient agar from which new plugs can be removed and subsequently placed onto fresh deficient agar for growth response measurements. Inoculum of many fungi can be prepared by using a shake-culture in which mycelium will pelletize. (See reference 75).

Dry weights can be determined by several methods. If no reproductive forms develop and little fragmentation occurs in any experimental treatment, J. G. Palmer and E. Hacskaylo use the following method: 1) collect mycelium from a single replicate on a fine mesh screen placed on a funnel, 2) wash with distilled water, 3) remove mycelium when liquid no longer drips through, 4) mold into wad with the top of the mycelial mat to the outside, 5) place on a blotter with or without an underlying screen, 6) move frequently (every 30 seconds initially) as moisture diffuses through the blotter until uniform solidification occurs, 7) place in a beaker, 8) dry in a forced-air oven at 90°C for 34 hours, 9) cool to room temperature in a dessicator over dry dessicant, and 10) weigh. If there are many colonies or mycelial fragments, mycelium must be collected on pre-washed, pre-numbered, pre-dried, and pre-weighed spun-glass filter papers. We mount them in a Buchner funnel and apply a vacuum during washing and drying before placing in the oven.

PURE CULTURE ECTOMYCORRHIZA SYNTHESIS

Melin (34, 35, 36, 38) developed the pure culture synthesis technique to experimentally demonstrate the ability of known fungus isolates to form ectomycorrhizae with specific hosts under pure culture conditions. His pioneering studies were instrumental in establishing which major taxa of higher fungi were involved in these associations. This information provided a framework for predicting ectomycorrhizal host-fungus associations based on field observations of sporocarp-host associations (see Trappe, 67). Use of the pure culture synthesis technique has also led to discovery of important physiological aspects of the symbiosis, including uptake of nutrients and water by the fungus and translocation to the host (5, 42, 44), movement of photosynthate from host to fungus (43), interactions of growth regulating substances (12, 64), host to host transfer of carbohydrates via a shared fungal symbiont (60), protection against root pathogens (29), effects of temperature on mycorrhiza development (30), specificity and compatibilty between fungus and host (50, 51, 52), and several other processes. This basic technique will continue to provide new information on the development and function of ectomycorrhizal symbioses.

One must recognize the artificiality of the pure culture synthesis and limit extrapolation of results to natural situations. Positive synthesis results are conclusive and confirm the ability of that particular host-fungus combination to form ectomycorrhizae. Negative results, however, are not conclusive in

themselves, but do suggest that the union of organisms in question seems unlikely.

Synthesis apparatus.--Size, arrangement, and complexity of the synthesis apparatus will depend upon the purpose of investigation. Melin (34, 35, 36, 38) primarily used flasks containing sterile sand moistened with a nutrient solution into which an aseptically germinated seedling and single fungus culture were introduced. Several investigators have modified Melin's techniques, often trying more complex arrangments to reduce the artificial nature of the enclosed system (17). With increased complexity also comes increased risk of contamination. Hacskaylo (13) greatly improved the system by using vermiculite instead of sand as the substrate; vermiculite provides better aeration and moisture holding capacity than sand. Marx and Zak (32) further improved the substrate by stabilizing the acidity with an addition of finely ground sphagnum peat moss. For a 2-liter flask culture, Zak (78) mixed 840 ml of vermiculite with 60 ml of finely ground peat moss in the flask, moistened this substrate with 550 ml of MMN nutrient solution, capped the flask with an inverted glass having a cheesecloth-wrapped cotton plug cemented to the bottom (Fig. 2), and autoclaved the assembly for 45-60 min at 120°C. Final pH is around 5.0. Molina (50, 51, 52) reports excellent seedling growth and ectomycorrhiza development in the following modified system: a 300 X 38 mm glass test tube is filled with 110 ml of vermiculite and 10 ml of peat moss, moistened with 70 ml of MMN nutrient solution, capped with an inverted 50 ml glass beaker, and autoclaved for 15 min at 120°C (Fig. 3). This arrangement allows for numerous syntheses to be run simutaneously in a relatively small area (Fig. 3). Pachlewski and Pachlewska (56) also reported good mycorrhiza syntheses in large test tubes but used a solid agar substrate rather than peat moss and vermiculite. Several alternative synthesis apparati and arrangements have been developed for specific experimentation but will not be described here (See references 2, 7, 17, 57, and 68).

Preparation of fungi.--Fungi are introduced into the synthesis vessel either as agar culture transfers or from liquid cultures. Agar cultures are first grown on nutrient agar in Petri plates for 3 to 4 weeks. Two to 4 agar plugs, 5-8 mm diam, are then aseptically removed and placed 1 to 2 cm deep around the germinant in the synthesis vessel. Liquid cultures are prepared by growing agar culture transfers in screw-capped glass prescription bottles containing 150 ml of sterile nutrient solution (MMN) with small bits of broken glass (about 1 cm deep) in the bottom. Liquid cultures are grown for 3 to 6 weeks at 20°C and hand shaken weekly to fragment the actively growing mycelium. Five to 10 ml of the liquid culture are then aseptically transferred into the synthesis vessel with a sterile, wide-bore pipette. Alternatively, the fungi can be grown in 20 X 150 mm screw capped glass test tubes filled with 10-15 ml of MMN solution and small bits of broken glass. After incubation and weekly shaking as described above, the glass lip of the tube is flamed and the entire liquid culture is aseptically poured into a single synthesis vessel. Because the liquid cultures are shaken just prior to inoculation, the numerous mycelial fragments will yield rapid and uniform colonization of the synthesis substrate.

Aseptic seed germination.--With minor modifications, the following procedure developed by Bratislav Zak (unpublished) for aseptic germination of Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) seed can be used for many ectomycorrhizal hosts. Select healthy seed, free of obvious defect, place them into a perforated plastic vial and treat as follows: 1) rinse in running tap water for 60 min; 2) wash in mild detergent solution (e.g., 2 to 3 drops of Tween 20 in 500 ml water) on a shaker for 90 min; 3) rinse in running tap water for 90 min; 4) soak in 30% hydrogen peroxide on a shaker for 60 min; 5) rinse aseptically with 2 liters of sterile distilled water; 6) shake excess water from vial and empty seed into a sterile Petri dish for planting.

Seeds are aseptically planted into nutrient agar in vials or plates. Planting should be done in a sterile transfer room or laminar flow hood when available. Incubate planted seed for 7-10 days at room temperature and check for contamination. Contaminated vials are discarded, or, if seeds are planted in plates, individually contaminated seed and the agar on which they rest can be cut out and removed, provided this is done before contaminants sporulate.

Figs. 2,3. Pure culture synthesis apparati. 2, Standard 2-liter flask culture with 4 mo. inoculated ponderosa pine seedling. 3, In situ arrangement of 38 x 300 mm glass synthesis tubes with bases submersed in cool tap water.

Noncontaminated seed are refrigerated (3-5°C) for 30 days (if stratification is needed) and then placed under artificial light at room temperature to germinate. The following soak periods in 30% hydrogen peroxide work well for other host species: Pinus spp., 30-60 min (66); Tsuga heterophylla (Raf.) Sarg. and Picea sitchensis (Bong.) Carr., 30 min (66); Larix occidentalis Nutt., 45 min; Eucalyptus spp., 10-30 min (25); Arbutus menziesii Pursh., 20 min (78); Arctostaphylos spp., 3 hr soak in concentrated sulfuric acid followed by 30 min in 30% hydrogen peroxide (77); Alnus species, 15 min (50, 51). Many host species will also germinate well without cold treatment. Surface sterilization with 30% hydrogen peroxide does not work well with hosts having resinous or pitchy seedcoats such as Abies spp.

Germinants are ready when radicles are approximately 1 to 2 cm long and cotyledons are still within the seed coat; germinants with longer radicles are difficult to plant. Use a hooked transfer needle with a long sterilizable handle for planting. Prepare a small planting hole by pushing aside some of the surface substrate. Aseptically transfer the germinant into the synthesis vessel so that the entire radicle is inserted in the substrate. Cover the seedcoat with a few mm of substrate. The fungus can be inoculated at this time for hosts that grow vigorously, such as Pinus or Pseudotsuga spp. Host species that grow slowly during the first month, e.g. Alnus, Tsuga, and Picea spp., are best inoculated 1 month after planting so that seedlings can develop adequately to cope with fungus competition. Because planting and inoculation necessitate exposing sterile synthesis vessels to the air for considerable periods, both procedures should be carried out in an air filtered transfer room or flow hood when available to reduce contamination risk.

Environmental set-up.--Several environmental set-ups can be used for pure culture syntheses, but two conditions are of prime concern. First, the area should be very clean and have a minimum of air turbulence to lessen the chance of contamination. Second, a build-up of heat within the synthesis vessel (greenhouse effect), particularly in the rooting substrate, should be avoided. At the Forestry Sciences Laboratory in Corvallis, Bratislav Zak designed a simple laboratory set-up for pure culture syntheses, consisting of a tank 15 cm deep filled 12 cm deep with constantly circulating unheated tap water and illuminated by an overhead combination of 35 watt fluorescent tubes and 25 watt incandescent

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bulbs (ca. 10500 lx) set for a 15-hr day (Fig. 3). The water bath cools the substrate. Standard growth chambers work well for mycorrhiza syntheses but special attention must be paid to contamination by rapidly circulating air and temperature control. Syntheses are also frequently carried out in greehouses, but the large fans commonly used for cooling present high contamination risk; added protection of the seal is needed under these conditions.

Synthesis evaluation.--Typical ectomycorrhiza syntheses are completed after 4 to 6 months, depending on the growth rates of fungus and host. If glass test tubes or agar substrates are used, ectomycorrhiza formation can often be seen directly through the glass walls. After a synthesis attempt is completed, a small bit of substrate should be aseptically removed from the vessel, transfered onto nutrient agar, incubated, and checked for contamination and reisolation of the original fungus. The seedling is removed intact from the synthesis vessel and its roots gently washed free of substrate with tap water. The entire root system is then placed under water in a Petri dish and observed with a stereomicroscope for ectomycorrhiza formation and characters. Specific observations should be recorded on the relative abundance of ectomycorrhizae present, mantle color and texture, presence of rhizomorphs and hyphal strands, length and branching pattern of ectomycorrhizae, and the overall size and health of the seedling. Suspected ectomycorrhizae are then sectioned by hand or microtome to characterize the mantle tissue and, most importantly, to confirm the presence and extent of Hartig net development. Any unusual or unique characters should also be noted. Root systems are easily stored in FAA for later sectioning and observations. Recording of other data may be important, depending on the investigator's purpose. Ectomycorrhizae are best photographed fresh.

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